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IL-6 promotes endometrial cancer cells invasion and migration through signal transducers and activators of transcription 3 signaling pathway

Qi Che[a], Xirong Xiao[b], Miao Liu[a], Yongning Lu[a], Xi Dong[a,*], Suying Liu[a,*]

[a] Reproductive Medicine Center, Zhongshan Hospital, Fudan University, Shanghai 200032, China
[b] Department of Obstetrics, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China

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ABSTRACT

Interleukin (IL)-6 is the most well-known traditional activator of activating signal transducers and activators of transcription 3 (Stat3). They have been proved to promote cancer progression in several human cancers. However, their exact roles in endometrial cancer have not been elucidated clearly. In this study, we aimed to investigate the role of IL-6/Stat3 signaling pathway in human endometrial cancer cells invasion and migration. We demonstrated that Stat3 is activated in endometrial cancer cell lines. To investigate the role of Stat3 in endometrial cancer invasive capacity, we used Stat3 inhibitor Stattic and found that Stattic significantly inhibited the migration and invasion of endometrial cancer cells elevated by IL-6. Furthermore, we showed that Stat3 inhibitor significantly decreased the expression of MMP2 enhanced by IL-6, indicating that IL-6 promoted endometrial cancer invasion and migration by Stat3-induced MMP2 upregulation. Taken together, our findings indicate that targeting IL-6/Stat3 pathway might be a potentially effective therapeutic strategy for treating endometrial cancer.

1. Introduction

Endometrial cancer is the most common malignant tumor of the female genital tract, and its prevalence is increasing [16]. Over the past 15 years, the rate of increase in mortality from endometrial cancer has accelerated faster than the incidence of the disease. The phenomenon is associated with the highly invasive growth of endometrial cancer [8]. Therefore, it is urgently needed to investigate the mechanism that contribute to the invasion and migration of endometrial cancer cells.

Dysregulated IL-6 family cytokine expression and downstream receptor signaling are frequent events in cancer and are often associated with poor clinical outcomes [17]. IL-6 family cytokines are now regarded as major therapeutic targets for clinical intervention [11]. Previously, we demonstrated that IL-6 was involved in the local estrogen biosynthesis process in endometrial cancer [4] and promoted endometrial cancer proliferation through an expanded autocrine regulatory loop [5]. Accumulating data show that IL-6 plays important roles in the promoting invasion of many different human cancers [11]. However, whether IL-6 is involved in the invasion and migration is not elucidated clearly.

In present study, we proved that IL-6 might promoted endometrial carcinoma invasion and migration through activating the Stat3 signaling pathway, which involved in an increased MMP2 expression. These data indicated that IL-6 to be important therapeutic targets in blocking the progression of endometrial carcinoma.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human IL-6 was purchased from Peprotech (Rocky Hill, NJ). Anti-total Stat3, anti-pStat3-Tyr705, anti-pStat3-Ser727, anti-MMP2 antibodies were from Epitomics (CA). Stat3 inhibitor V Stattic were from Selleck Chemicals (Houston, TX).

2.2. Cell culture

Human endometrial carcinoma cell lines, Ishikawa and RL95-2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to the provider's instruction in DMEM/F12 (Gibco, Auckland, NZ) supplemented with 10% fetal bovine serum (FBS).

2.3. Immunofluorescence

Cultures growing on chamber slides or were fixed in 4%

* Corresponding authors at: Reproductive Medicine Center, Zhongshan Hospital, Fudan University, No. 250, Xiaomuqiao Road, Shanghai 200032, China.
E-mail addresses: dong.xi@zs-hospital.sh.cn (X. Dong), lsy6592@163.com (S. Liu).

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paraformaldehyde and permeabilized with 0.2% Triton X-100 for 5 min. Cells were incubated with the diluted primary antibodies for Stat3 for overnight at 4℃. Cells were then treated with tetramethyl rhodamine isothiocyanate-conjugated or fluorescein isothiocyanate–conjugated secondary antibody (BD Biosciences, San Jose, CA) for 1 h. Nuclei were visualized by counterstaining with DAPI. Samples were then analyzed using a fluorescence microscope (Leica DMI 3000B, Solms, Germany). The control slides received PBS in place of the primary antibody.

2.4. Wound-healing assay

Ishikawa and RL95-2 cells were seeded to nearly complete confluence in a monolayer in 24-well plates. Before stimulation with IL-6 (20 ng/ml) and preincubation for 1 h with Stat3 inhibitor Stattic (20 μM), cells were serum starved overnight. Then a lesion was created using a plastic pipette tip, and the cells were washed three times with PBS to remove the debris. The monolayer was cultured with serum and phenol red-free DMEM/F12 medium at 5% CO_2 and 37 ˚C, and photographs were taken at the designated time (12 h and 24 h) by an inverted microscope (Olympus, IX71, Tokyo, Japan).

2.5. Transwell migration assay

Ishikawa and RL95-2 cells were stimulated with IL-6 (20 ng/ml) in the presence or absence of Stat3 inhibitor V Stattic (20 μM) for 24 h and starved for 12 h in DMEM/F12 media with 0.1% FBS. Then cells were trypsinized and resuspended into DMEM/F12 containing 0.1% bovine serum albumin. Approximately 2×10^5 cells in serum-free media were added to the top chamber of 24-well transwell plates (Corning, 8-μm pore size), and DMEM/F12 containing 10% FBS was added to the bottom chamber. After 24 h of incubation, top cells (nonmigrated) on the upper surface were gently removed with a cotton swab and bottom cells (migrated) were fixed in 4% methanol for 30 min and stained with a 0.1% crystal violet solution for 30 min. The number of migrating cells in five fields was counted, and the means for each chamber were determined. Experiments were repeated three times.

2.6. Western blot

For western blot analysis, cells were lysed in lysis buffer for 30 min at 4 ˚C. Total proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were then incubated with appropriate primary antibodies (total Stat3, pStat3-Tyr705, pStat3-Ser727, MMP2 and β-actin), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). The probed proteins were detected by enhanced chemiluminescent reagents. β-actin was used as an internal control.

2.7. Total RNA extraction and real-time reverse transcription- PCR

Total RNA from Ishikawa, RL95-2 cells was isolated by Trizol (15596-026, Invitrogen) and cDNA was prepared using the reverse transcriptase kit. Real-time reverse transcription (RT)-PCR was conducted using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) and performed with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). A comparative CT method ($2^{-\Delta\Delta CT}$) was used to analyze the relative changes in gene expression. The results are expressed relative to the number of GAPDH transcripts (internal control). For MMP2 mRNA real-time RT-PCR, the forward and reverse primers were 5′- TGATCTTGACC AGAATACCATCGA-3′ and 5′-GGCTTGCGAGGGAAGAAGTT-3′, and for GAPDH mRNA (control), the forward and reverse primers were 5′-GAAGGTGA AGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

2.8. Enzyme-linked immunosorbent assay (ELISA)

MMP2 protein levels were detected in culture medium using solid phase sandwich ELISA assays according to the manufacturer's protocol (R&D Systems). The MMP2 assay sensitivity was 0.7 pg/ml, and the assay range was 3.12–300 pg/ml. For the statistical analysis, culture medium was collected three times independently.

2.9. Statistical analysis

Continuous variables were recorded as mean ± SD and analyzed with the Student's *t*-test. All statistical analyses were done using Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, IL). P values < 0.05 were considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Stat3 activation was observed by stimulated with IL-6 in endometrial cancer cells

To observe the activation of Stat3 induced by IL-6, we examined the expression and the localization of Stat3 stimulated by IL-6 in Ishikawa and RL95-2 cells. Using immunofluorescence, Stat3 immunoreactivity in plasma was observed in untreated cells and Stat3 nuclear translocalization was observed after IL-6 treatment for 24 h (Fig. 1).

3.2. IL-6 regulated the migration and invasion of endometrial cancer cells through Stat3 signaling pathway

To demonstrate the potential effects of IL-6 on Ishikawa and RL95-2 cells, we performed tumor cell migration and invasion assays. As shown in the results of scratch wound healing assays, IL-6 could increase cell migration ability in Ishikawa and RL95-2 cells after IL-6 incubation for

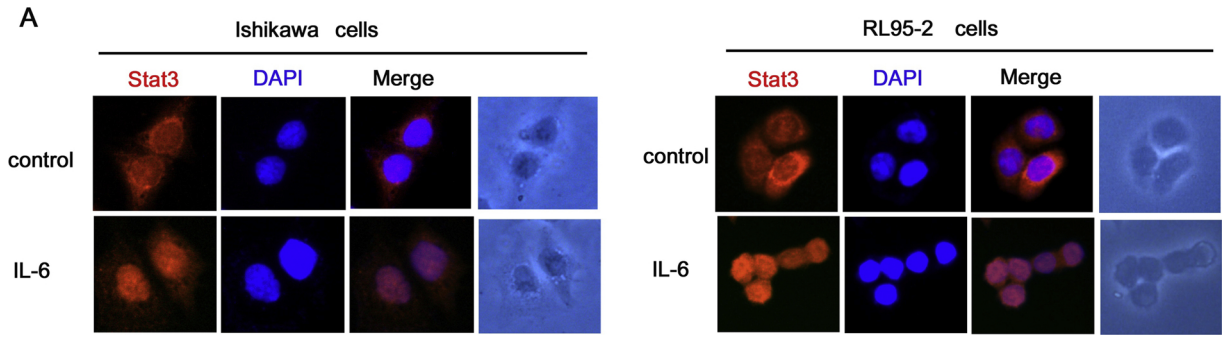

Fig. 1. Immunofluorescence images of Ishikawa and RL95-2 cells showing the nuclear translocalization of Stat3 (*red*) after IL-6 incubation. *Blue*, DAPI-stained nuclei. Original magnification ×200. The right line is photograph of cells morphology.

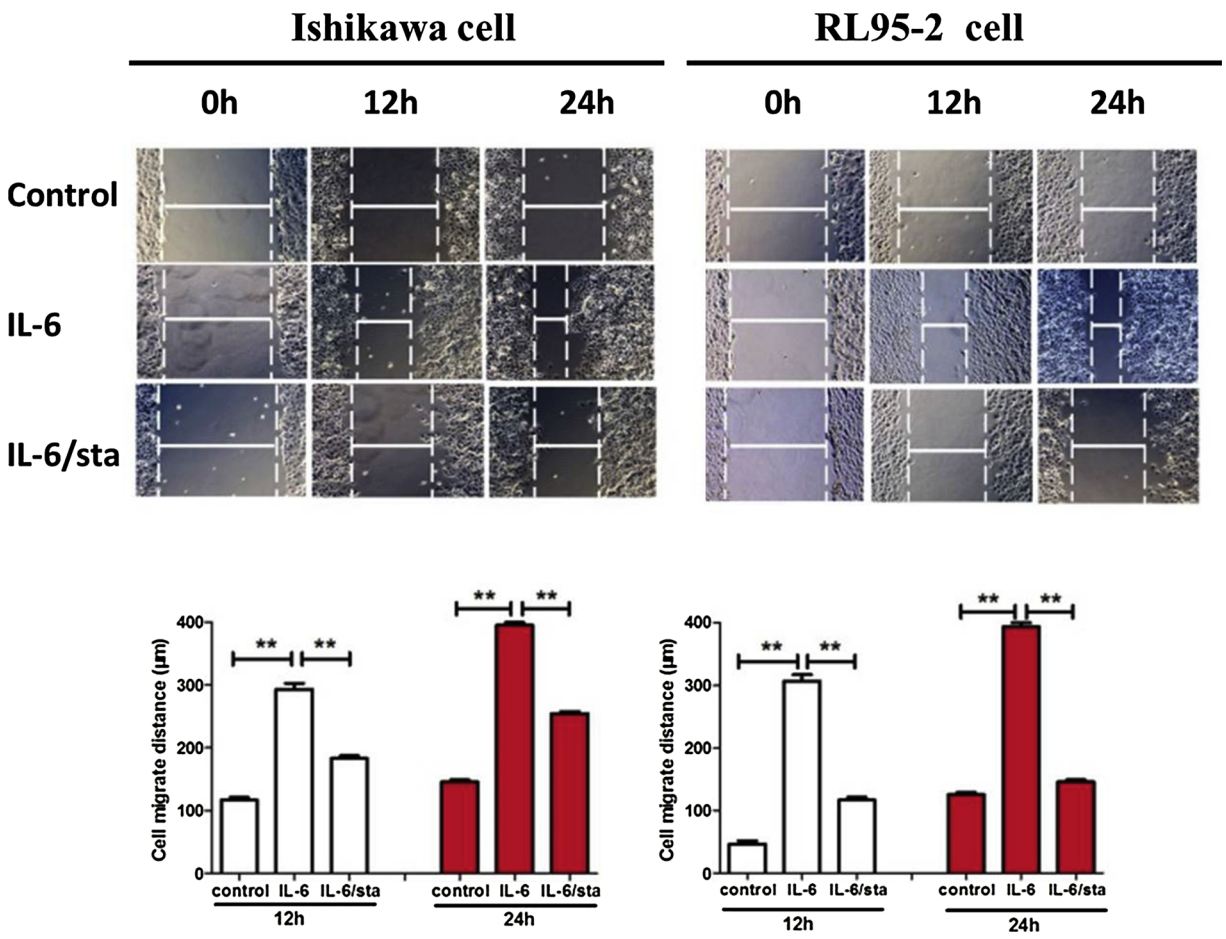

Fig. 2. Ishikawa and RL95-2 cells treated with IL-6 demonstrated increased wound recovery at 12 h and 24 h after wounding, which was attenuated by Stattic. IL-6/sta, IL-6 with 20 μM Stattic. $^{**}P < 0.01$ Experiments were repeated three times.

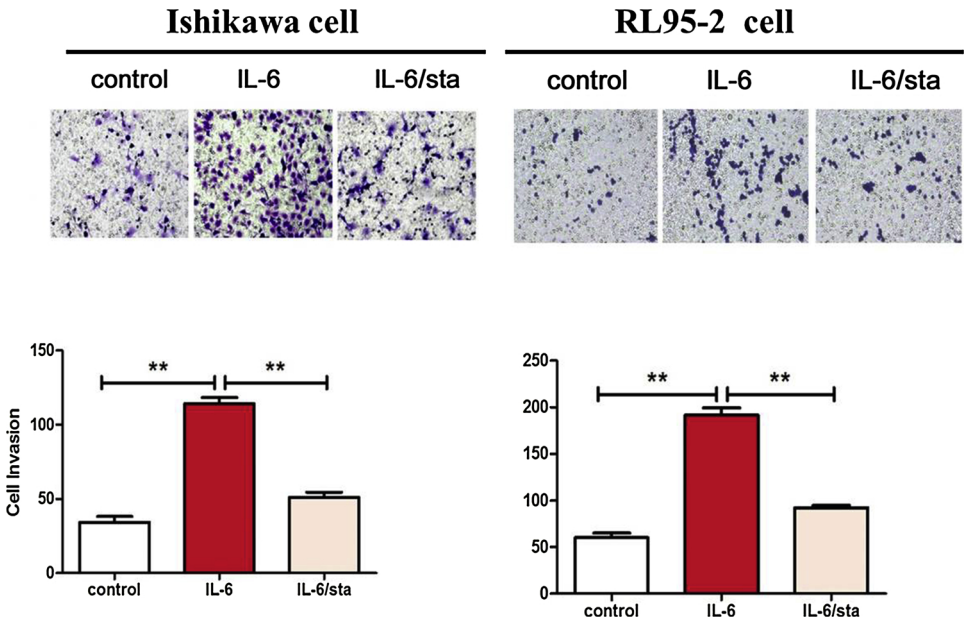

Fig. 3. Transwell assay results showed that IL-6 promoted cell invasive capacity of Ishikawa and RL95-2 cells, which was inhibited by Stattic. IL-6/sta, IL-6 with 20 μM Stattic. $^{**}P < 0.01$. Experiments were repeated three times.

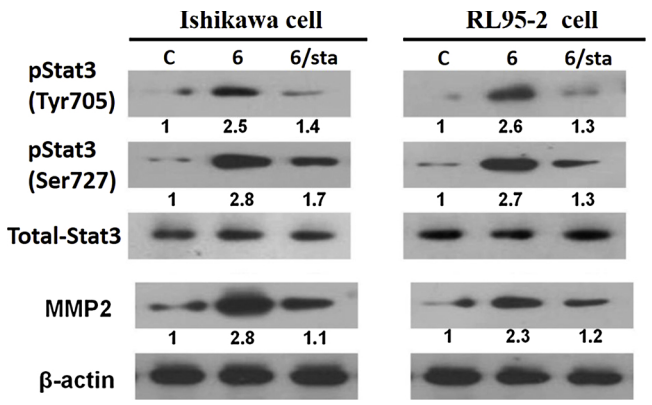

Fig. 4. Western blot analysis to measure the phosphorylated and total Stat3 levels and MMP2 expression in Ishikawa and RL95-2 cells after IL-6 treatment, in the present or absent of Stattic. IL-6/sta, IL-6 with 20 μM Stattic; T-Stat3, total Stat3.

12 h and 24 h compared to the control group, and the enhancement was suppressed by Stattic as shown in Fig. 2. In the transwell migration assay, IL-6 elevated the numbers of Ishikawa and RL95-2 cells that migrated to the bottom chamber compared to the control group, and Stattic could partially inhibit the elevation (Fig. 3). From these results, we indicated that blocking Stat3 signaling pathway may inhibit the migration and invasion of endometrial cells induced by IL-6.

3.3. IL-6 induced the Stat3 phosphorylation and MMP2 expression

To gain insight into the mechanism underlying the downstream pathway of IL-6, we next examined the activation of Stat3 stimulated by IL-6 using western blot. An increase in Stat3 phosphorylation, including pStat3-Tyr705 and pStat3-Ser727 was observed when cells were stimulated by IL-6, whereas simultaneous treatment with Stattic reduced the upregulation. Immunoblots were reprobed with antibodies against total Stat3, showing that the increase in Stat3 phosphorylation was not due to the increased protein expression. Furthermore, we detected the effect of IL-6 on expression of MMP2. As shown in Fig. 4, incubation with IL-6 induced MMP2 protein expression in Ishikawa and RL95-2 cells, while in the present of Stattic, the enhanced expression of the protein was partially reduced. The MMP2 protein concentration in the culture medium was measured using ELISA and MMP2 mRNA expression was detected by realtime-PCR. The upregulation of MMP2 was also observed in the IL-6 treated group and partially suppressed by Stattic (Fig. 5).

4. Discussion

Prognosis of endometrial cancer is dependent on many factors including time of diagnosis, progression of tumor differentiation, and degree of invasion and metastasis [15]. Stat3 is constitutively activated in a number of human tumors, including endometrial cancer [6]. During malignant transformation, Stat3 is frequently overexpressed and constitutively activated by phosphorylation, which is why it has attracted much attention as a potential pharmacologic target for treatment [13].

In the present study, we sought to determine whether the IL-6/Stat3 signaling pathway regulates the invasive and migrated potential of endometrial cancer cells. Stat3, a member of the Janus kinase (JAK)/Stat signaling pathway, is a central cytoplasmic transcription factor that is activated by phosphorylation in response to extracellular signals and oncogenes. Activation of STAT3 is by JAK2-dependent phosphorylation at the tyrosine-705 (Y705), and JAK2-independent phosphorylation mediated by mitogen-activated protein kinases (MAPKs)at the serine-727 (S727) position [1]. Once phosphorylated, two Stat3 monomers form dimers and translocate to the nucleus where they bind to Stat3-specific DNA response elements of target genes, and induce gene transcription [22]. In Ishikawa and RL95-2 cells, incubation with IL-6 resulted in an increase in nuclear Stat3 staining, which indicated that it was activated by stimulation of IL-6.

Furthermore, we found that IL-6 correlates with the invasion and migration capacity of the endometrial cancer cell lines. Inhibition of Stat3 by Stattic not only significantly suppressed cell migration capacity, but also reduced invasive potential of endometrial cancer cells. It has been observed that aberrant Stat3 activation in tumor cells is associated with cell proliferation, cell survival, invasion, and metastasis [9,12]. Conversely, inhibiting Stat3 activation could reduce tumor growth and invasion both *in vitro* and *in vivo* without affecting normal cells, suggesting that Stat3 could be a valid molecular target for cancer therapy [3]. Once activated, Stat dimerizes to other Stats by reciprocal SH2 phosphotyrosine interaction, leading to its translocation into the nucleus followed by its binding to the specific enhancer elements for initiation of transcription [22]. Since the function of the Stat3 SH2 domain is crucial for Stat3 activation, Stat3 signaling might be inhibited by small molecules that impair the function of the Stat3 SH2 domain. Stattic inhibits binding of a physiologically relevant phosphorylated peptide motif to the Stat3 SH2 domain, potently and selectively inhibiting Stat3 activation and nuclear translocation [18]. Stat3 is excessively active in many cancers and plays a central role in tumorigenesis. Several lines of evidence have implicated that inhibition of Stat3 attenuates the proliferation and survival of a wide variety of cancers with little or no effects on normal cells. Our results about the Stat3 inhibitor have validated Stat3 as a target for endometrial cancer therapy [12].

Invasion to extracellular matrix is one of the key steps in tumor growth and metastasis formation [2]. Cellular migration is a central step for many biological processes, including embryogenesis, cell invasion, and cancer metastasis [10]. Cumulative evidence has indicated Stat3 plays a crucial role in cellular migration under normal as well as pathological conditions [20]. Many studies strongly suggest that Stat3 is involved in this complex multistep process by regulating the MMPs [12]. In particular, MMP-2, a 72-kDa type IV collagenase, also referred to as gelatinase A, has been shown to be one of the key enzymes in the invasion and metastasis cascade of malignant melanoma [21]. MMP2 expression is regulated by various cytokines and growth factors, including IL-6 [14,23], and by oncogenic proteins such as Src [19] and Ras [7]. Significantly, many tumors often exhibit overexpression of these molecules; and these molecules often transmit signals through Stat3 [22]. Therefore, it is speculative that Stat3 activation is a common signaling intermediate leading to the overexpression of MMP2 in malignant tumors. Our results demonstrated that activation of Stat3 signaling pathway was crucial for upregulation of MMP2 in endometrial cancer cells and inhibition of Stat3 may offer a useful strategy for MMP2 intervention.

5. Conclusion

Taken together, we explained the mechanisms of IL-6 in the invasion and migration process of endometrial cancer and provided evidence for the critical role of constitutively activated Stat3 and its target gene MMP2 in tumor progression. Meanwhile, we identify Stat3 inhibitor to interfere with IL-6/Stat3 signaling for potential therapeutic intervention in endometrial cancer. It provides the molecular and pharmacological strategies for endometrial cancer treatment.

Conflicts of interest

The authors declare no conflict of interest.

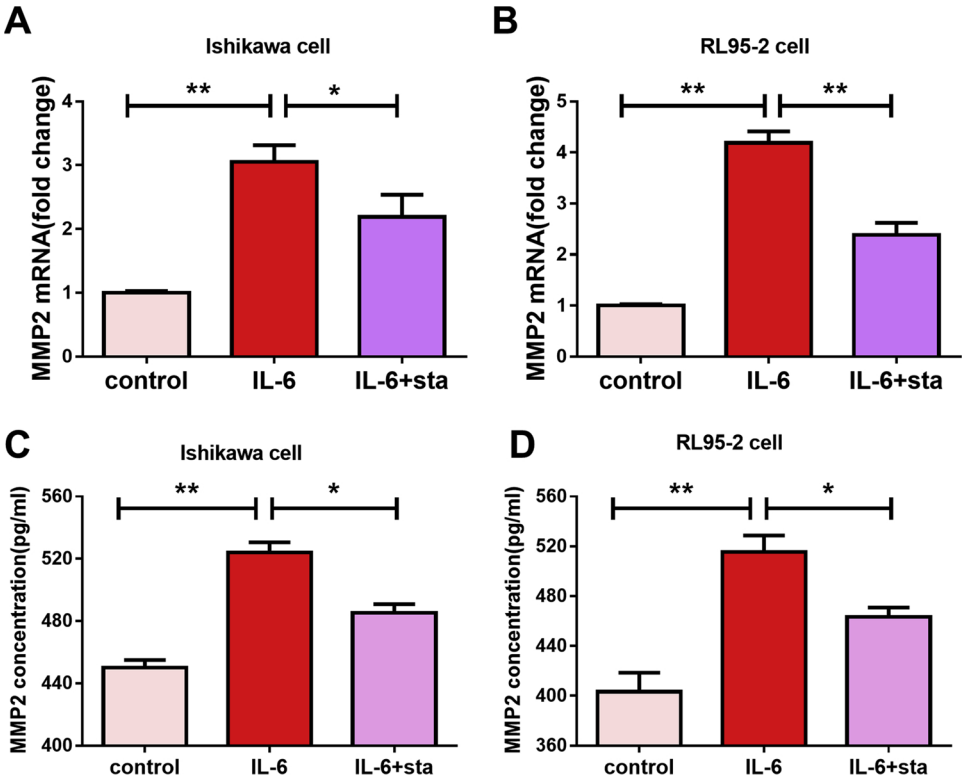

Fig. 5. MMP2 mRNA expression (A and B) and protein concentration (C and D) were increased by treated with IL-6 in Ishikawa and RL95-2 cells culture media and attenuated by Stattic. $^{*}P < 0.05$, $^{**}P < 0.01$.

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